Applicants reserve their rights to file divisional application directed to the cancelled subject matter. Claims 23 to 25 have also been amended to clarify the present invention. Applicants submit that no new matter has been added via the amendment to the specification. Claims 23 to 26 are pending.

The examiner has pointed out several informalities. Applicants submit that some of these informalities have been addressed. Claims 27 and 28 have been cancelled as being drawn to a non-elected invention. The specification has been amended to insert the status of the present application. While the drawings have been objected to under 37 C.F.R. 1.84, Applicants will respond to this objection upon allowance of this application.

Claims 23-26 have been objected to for alleged lack of clarity. Therefore, Claims 23-25 have been amended to conform with the examiner's suggestion. Therefore, this objection should be rendered moot.

Claims 23-25 have been rejected under 35 U.S.C. § 101 since the claimed invention was directed to non-statutory subject matter. In order to obviate the rejection, the claims have been amended to recite "An isolated peptide or polypeptide", thereby rendering this rejection moot.

Claims 23-26 have been rejected under 35 U.S.C. § 112, first paragraph, because there was no Declaration of availability concerning the microorganisms. Enclosed please find an executed Declaration of availability. Therefore, this rejection is rendered moot.

Claim 23 has been rejected under 35 U.S.C. § 112, first paragraph, allegedly because it was not clear to the examiner, from the results of Table 1, which amino acid sequence the monoclonal antibody, 64G12, is reacting with. For the following reasons, this rejection is respectfully traversed.

In Example 1 (page 14), the application discloses the construction of a plasmid for the expression in <u>E. coli</u> of the extracellular domain (amino acid 27 to 427) of the human IFN-R (figure 2). It is clearly specified that this plasmid was introduced in the <u>E. coli</u> strain, JM 105.

At least on page 16, the construction of another expression vector coding for the IFN-R amino acid sequence 1-427, with an additional 5-histidyl residues at the C-terminus, is disclosed. This second plasmid was introduced into Cos7 cells.

Therefore, the E. coli cells mentioned in Table 1 express the IFN-R 27-427 fragment, whereas the Cos cells in the same table express the IFN-R 1-427 fragment.

Hence, it is clear from the results shown in Table 1 that the monoclonal antibody, 64G12, is reacting with both the 27-427 and 1-427 fragments.

Thus, in view of the above, withdrawal of this rejection is respectfully requested.

Claims 24 and 25 have been rejected under 35 U.S.C. § 112, first paragraph, allegedly because there was no evidence of record that peptides or polypeptides consisting of the amino acid sequence 27-229 or 1-229 of SEQ ID No: 1 or 2 would bind specifically to the monoclonal antibody, 64G12. Enclosed please find a Declaration under 37 C.F.R. § 1.132, by Michael G. Tovey, where the fact that these peptides or polypeptides bind specifically to the monoclonal antibody, 64G12, is illustrated.

Therefore, in view of the above, withdrawal of this rejection is respectfully requested.

Claims 23-26 have been rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains or with which it is nearly connected, to make and/or use the invention. However, for the following reasons, this rejection is respectfully traversed.

The instant claims recite "a portion" of a peptide or polypeptide consisting of the amino acid sequences 27-427, 27-229 or 1-229 of SEQ ID No: 1 or 2, or an analogue of the amino acid sequence 27-427, each binding specifically to the monoclonal antibody, 64G12. The examiner asserts that the specification "is not enabled for such "a portion" of any of the amino acid sequence recited, and for their binding specificity with the monoclonal antibody, 64G12." See Office Action mailed September 3, 1999, page 6, last sentence. The examiner says that there is no evidence that such "a portion" consisting, for example, of two amino acid residues would bind specifically to the monoclonal antibody, 64G12.

It is respectfully submitted that it is obvious to one skilled in the art that a polypeptide must be longer than merely two amino acids to be recognized by an antibody. Taking into account the fact that the monoclonal antibody, 64G12, is available, Applicants respectfully submit that enough specific guidance is provided in the specification and the examples to permit a person skilled in the art at the time of the effective filing date of the instant application to reproducibly practice the invention as claimed. Indeed, the artisan skilled in the art only had to produce a set of overlapping peptides and test their binding to 64G12, as explained, for example, in the enclosed Declaration. This kind of experiment is classically used in the field of the invention, for the mapping of epitopes. It is therefore known by the skilled artisan.

The necessary experiments are merely routine, and thus are not undue experimentation, as explained in *Ex porte Jackson* 217, USPQ 804 (Bd. Pat. App. 1982):

> "The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed to enable the determination of how to practice a desired embodiment of the invention claimed . . ."

Therefore, in view of the above, withdrawal of this rejection is respectfully requested.

Claim 26 has been rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains or with which it is nearly connected, to make and/or use the invention. However, for the following reasons, this rejection is respectfully traversed.

The instant claims recite an "analogue" of a peptide or polypeptide consisting of the amino acid sequences 27-427 of SEQ ID No: 1 or 2, which retains the ability to bind specifically to the monoclonal antibody, 64G12. The examiner asserts that the specification is not enabled for such an "analogue" of the amino acid sequence recited and for their binding specificity with the monoclonal antibody, 64G12. The examiner says that there is no evidence that such an "analogue" would bind specifically to the monoclonal antibody, 64G12.

It is respectfully submitted that it is obvious to one skilled in the art that peptides or polypeptides such as those claimed in the present application comprise a particular epitope(s) that is recognized by the antibody, and that other parts of the molecule can be substituted without changing the binding affinity of said peptides or polypeptides with the antibody. Taking into account the fact that the monoclonal antibody, 64G12, is available, Applicants respectfully submit that enough specific guidance is provided in the specification and the examples to permit a person skilled in the art at the time of the effective filing date of the instant application to reproducibly practice the invention as claimed. Indeed, the skilled artisan only had to produce the desired mutant and to test its binding to the monoclonal antibody, 64G12.

The necessary experiments are merely routine, and thus are not undue experimentation, as explained in above-cited *Ex parte Jackson* (1982).

Therefore, in view of the above, withdrawal of this rejection is respectfully requested.

Claims 23-26 have been rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for falling to particularly point out and distinctly claim the subject matter which Applicants regard as the invention.

For the following reasons, this rejection is respectfully traversed.

The peptides or polypeptides claimed in Claims 23-20 are characterized by the two following characteristics:

(1) they are derived from (i.e., identical to, a portion of, or an analogue of) the extracellular portion of the IFN-R of SEQ ID NO: 2,

(2) they specifically bind to monoclonal antibody 64G12.

A peptide or polypeptide of the invention must have <u>both</u> of these elements. The presence of these elements is very simple to test, the first point being checked by sequencing and the second point by contacting said peptide or polypeptide with said monoclonal antibody, 64G12, and evaluating their binding.

Therefore, it is respectfully submitted that the scope of Claims 23-26 is clear enough to a person skilled in the art, and withdrawal of this rejection is hence requested.

Applicants wish to note that the examiner has not indicated his consideration of the prior art references submitted on August 2, 1999. Applicants request that examiner return

an appropriately initialed 1449 form. A copy of the submitted 1449 form is enclosed, along with a copy of the acknowledged receipt.

Applicants soon will forward copies the submitted declarations to replace the facsimile versions enclosed herein.

In view of the foregoing amendments and remarks it is believed that the application now is in condition for allowance. A favorable disposition of the application therefore is solicited. The examiner also is invited to contact the undersigned if there are any questions or if the examiner believes that further discussion will advance prosecution.

Respectfully submitted,

35,087

March 3, 2000
Date

Bernhard D. Saxe
Registration No. 28,665

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3000 K Street, N.W., Suite 500
Washington, D.C. 20007-5109
(202) 672-5300

PATENT

Attorney Docket No. 017283/0123

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	)
BENOIT et al.	)
Serial No.: 09/240,675	) Group Art Unit: 1641
Filed: 02/02/99	) Examiner: DEVI, S
For: MONOCLONAL ANTIBODY AGAINST ALPHA IFN	)

Declaration pursuant to 37 C.F.R. § 1.132

Hon. Commissioner of Patents
and Trademarks

Washington, D.C. 20231

I, Michael G. TOVEY, do hereby declare and state the following :

1. That I have received a Special Bachelor of Sciences in Microbiology in 1969 and a Ph.D. in Microbiology in 1972 from the University of London. I am currently Director of the Laboratory of Viral Oncology (UPR CNRS 9045) in Villejuif, France. I am or have been a member of the Editorial Boards of several reviews including the Journal of Interferon Research, Oncology Reports, and Cellular Pharmacology. I am also a member of the Scientific Boards of several organisations, particularly in the fields of Oncology and Immunogenetics. Enclosed, please find a copy of my *curriculum vitae*, which clearly indicates my expertise in the fields of Oncology and Immunogenetics.

2. I am one of the inventors of the above-captioned patent application and therefore I am very familiar with the subject application. I have read and understood the latest Official Action issued by the U.S. Patent and Trademark Office on September 3, 1999. It is my understanding that Claims 24 and 25 were rejected under 35 U.S.C. § 112, first paragraph, as not being reasonably enabled. In rendering this rejection, it appears that the Examiner deems that no evidence is of record that peptides or polypeptides consisting of the amino acid sequences 1-229 or 27-229 of SEQ ID NO: 1 or 2 would bind specifically to the monoclonal antibody, 64G12.

3. In order to address the issue of enablement concerning the subject-matter of Claims 24 and 25, the following experiments, were conducted under my supervision. A brief explanation of why these experiments were conducted and the results of these experiments are set forth below :

Interfons (IFNs) in common with other cytokines activate Janus tyrosine kinases and latent STAT transcription factors upon binding to their cell surface receptor. Type I IFNs bind to a receptor composed of two transmembrane polypeptides, IFNAR1 and IFNAR2, which belong to the class II cytokine receptor family that also includes the cellular receptors for IFN-γ, Interleukin-10 and coagulation protease factor VII (Tissue factor). The extracellular domain of the Type I IFN receptor chain IFNARI, has four fibronectin type-III sub-domains. Human IFNAR1 has intrinsic weak affinity for Type I IFNs and plays an essential role in transmembrane signaling, formation of a high affinity complex with IFN and the modulation of ligand specificity.

4. **Reactivity of the 64G12 mAb against various bovine/human IFNAR1 chimeras**

Human IFNAR1 is a cell surface glycoprotein, composed of a large extracellular region (457 amino acids), a single transmembrane domain (21 amino acids) and a cytoplasmic domain (100 amino acids). The extracellular region is divided into 4 subdomains separated by conserved di- or tri-proline motifs denoted SD1 to SD4. Although 66% of the amino acids are conserved between bovine and human IFNAR1, the 64G12 mAb does not recognize bovine IFNAR1. Bovine/human IFNAR1 chimeras, produced in transiently transfected COS cells, have proved to be a useful tool for mapping different mAbs agains IFNAR1 subdomains (Goldman et al., 1998, 37: 13003-13010). Thus, the cell surface reactivity of fluorescent labelled 64G12 mAb was assayed by flow cytometry against 8 different bovine/human chimeras encompassing the 4 subdomains (SD1 to SD4, see Table I). The results of these experiments clearly show, that the presence of the human SD1 (residues 28-123) subdomain, is an absolute requirement for the binding of the 64G12 mAb to the hybrid chimeras.

HHHH	+	BBBB	-
HBHH	+	BHBB	-
HHBH	+	BBHB	-
HHHB	+	BBBH	-
HBBB	+	BHHH	-

Table 1: Reactivity of the 64G12 mAb against various bovine/human IFNARI chimeras expressed transiently at high levels in COS cells.

The chimeras (H for human and B for bovine), represent the 4 extracellular subdomains (SD1-4), starting at the amino terminus (SD1: residues 28-123, SD2: residues 124-232, SD3: residues 233-335, SD4: residues 336-436)

5. IFNAR1-derived overlapping peptide scans for the mapping of the linear 64G12 epitope

The 64G12 mAb inhibits binding of the Type 1 IFNs to the transmembrane complex IFNAR1-IFNAR2 and competes specifically with IFN-α/β for cell surface receptor binding. Although the antibody is able to remove IFN bound to its cell surface receptor, IFN cannot remove the bound antibody. Thus, in order to determine the precise role of putative IFNAR1 target amino acids in IFN binding, epitope mapping of the site recognized by the monoclonal antibody 64G12 was performed.

In order to characterize the ligand binding site on IFNAR1, the epitope recognized by the IFN-α and IFN-β was analyzed. The target peptide recognized by the 64G12 mAb was determined by screening a set of 48 overlapping peptides covering the first two subdomains (residues 23-229) of the extracellular region of IFNAR1. The results of this study show that the peptide (FSSLKLNVY), localized within the first sub-domain, exposed thereafter, (residues 89-97) of IFNAR1 is recognized by the 64G12 mAb.

Mapping of the 64G12 linear epitope was carried out using IFNAR1-derived scans of overlapping 20-mer biotinylated peptides, prepared by solid phase synthesis, as a tool for probing the SD1 and SD2 domains (residues : 23-2[illegible]) of the human IFNAR1 ectodomain (Table 2). We chose biotinylated peptides as they exhibit a low level of non specific binding when detecting sequential epitopes. A number of observations suggested that a sequential epitope was present. Thus, the 64G12 mAb is able to recognize completely denatured IFNAR1 in lysates from a variety of different cell lines using Western blotting or immunoprecipitation. Similar results are obtained using deglycosylated recombinant IFNAR1 or whole transmembrane IFNAR1 from cell extracts. In addition, 64G12 was able to recognize native or denatured recombinant IFNAR1 produced in E. coli. A copy of an article (Eid and Tovey, 1995/J. Interferon Res./[illegible]: 205-211) is enclosed, which reports that the 64G12 antibody is directed against the first two subdomains SD1 and SD2 (amino acids 1-[illegible]), of IFNAR1. The most relevant passages are indicated (abstract and page 209).

The ELISA screening of the 48 biotinylated IFNAR1 peptides was performed on streptavidin-coated plates as described in the experimental procedures. The data, presented in Figure 3, indicate that the epitope recognized by the 64G12 mAb is located in the first subdomain (SD1) of the extracellular moiety of IFNAR1. Specific binding of the 64G12 mAb to the coated peptides was observed in the region corresponding to peptides 14-18. No specific binding was seen when an irrelevant mouse IgG1 was used to screen the streptavidin linked peptides (Figure 1, Table 2).

// SHOULD BE FIGURE 1 MBT. 02-03-00.

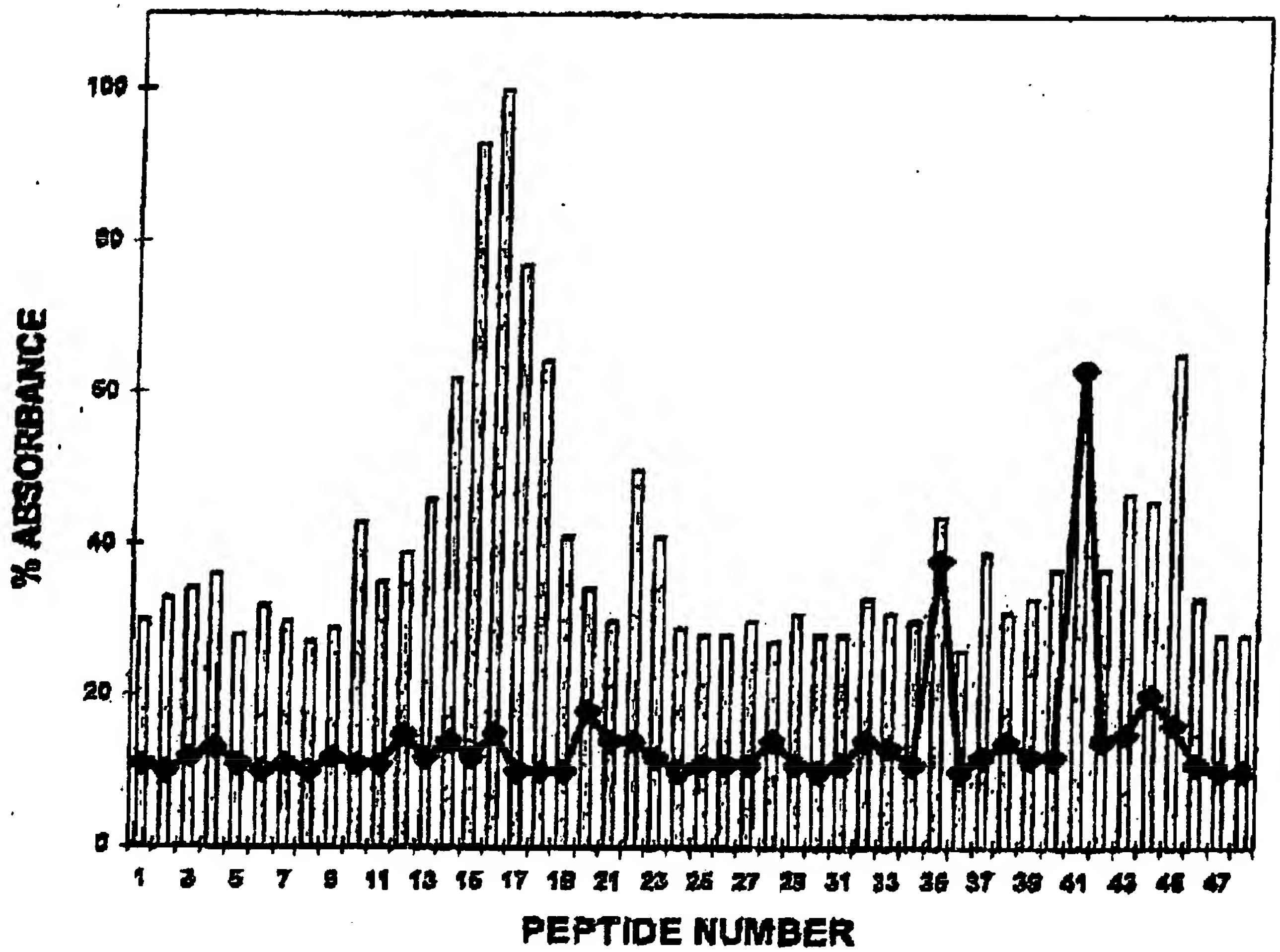

FIGURE 1. Binding of anti-IFNAR1 mAb (64G12) to overlapping peptides coated-plates. The monoclonal antibody 64G12 mAb was scanned in ELISA with a set of overlapping biotinylated 20 mer peptides, each offset by four residues, spanning the SD1, and SD2 subdomains (residues 23-237, Table 1) of the human IFNAR1 molecule. Peptides are numbered from 1 to 48 starting from the N-terminus. Histograms represent the binding of 64G12 for each coated peptide and the continuous line, the binding of an irrelevant mouse IgG1. The data presented are the average of two independent experiments. Values are the means of duplicate determinations.

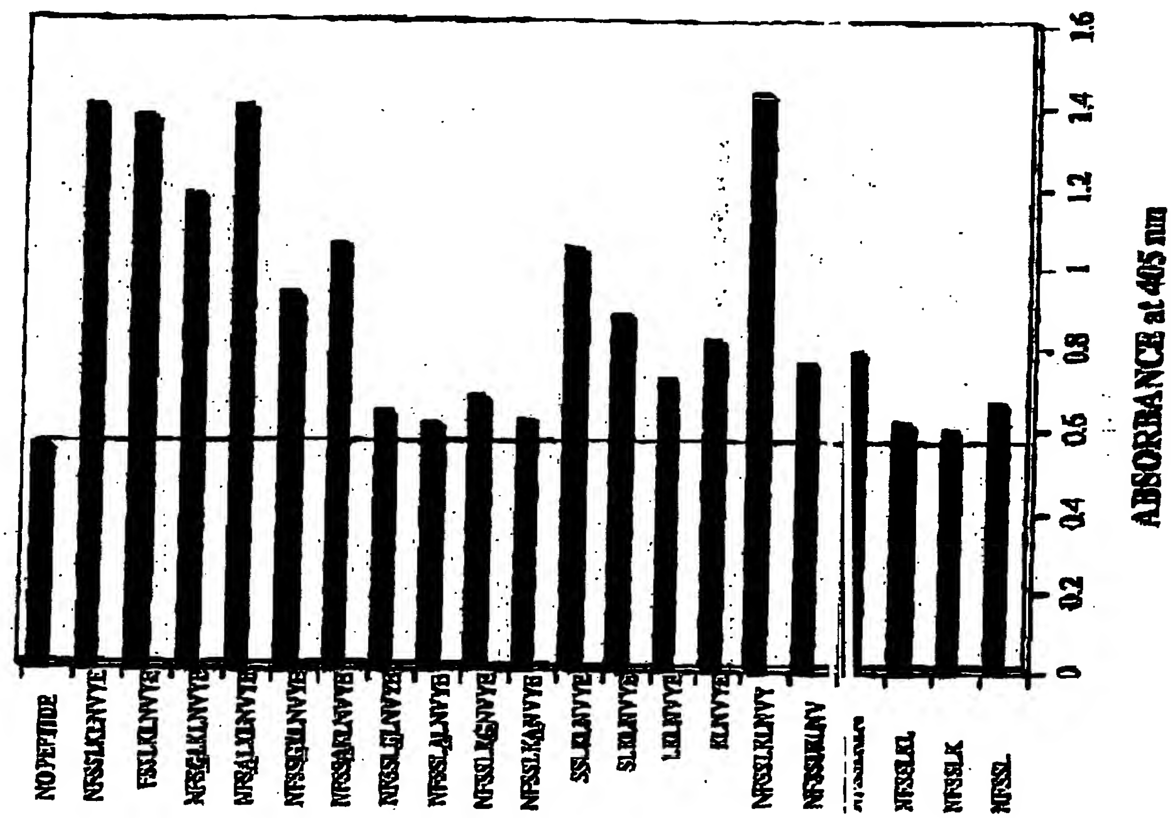
ABSORBANCE at 405 nm
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
NO PEPTIDE
NFSSLKL
NFSSLK
NFSSL

Fig 4

Peptide	Position	Sequence	M.W	pI	Binding %
1	23-42	AAASGKNLKSPQKVEVDIID	2052.6	6.36	30
2	27-46	GKNLKSPQKVEVDIIDDNFI	2271.8	4.49	33
3	31-50	KSPQKVEVDIIDDNFILRWN	2428.9	4.48	34
4	35-54	KVEVDIIDDNFILRWNRSDE	2475.9	4.03	36
5	39-58	DIIDDNFILRWNRSDESVGN	2377.8	3.79	28
6	43-62	DNFILRWNRSDESVGNVTFS	2355.7	4.26	32
7	47-66	LRWNRSDESVGNVTFSFDYQ	2419.7	4.36	30
8	51-70	RSDESVGNVTFSFDYQKTGM	2267.6	4.36	27
9	55-74	SVGNVTFSFDYQKTGMDNWI	2308.7	3.92	29
10	59-78	VTFSFDYQKTGMDNWIKLSG	2336.9	6.07	43
11	63-82	FDYQKTGMDNWIKLSGCQNI	2161.0	6.03	36
12	67-86	KTGMDNWIKLSGCQNITSTK	2224.9	9.05	39
13	71-90	DNWIKLSGCQNITSTKCNFS	2258.9	8.00	46
14	75-94	KLSGCQNITSTKCNFSSLKL	2172.0	9.19	62
15	79-98	CQNITSTKCNFSSLKLNVYE	2281.9	7.93	93
16	83-102	TSTKCNFSSLKLNVYEEIKL	2317.0	8.20	100
17	87-106	CNFSSLKLNVYEEIKLRIRA	2396.2	9.06	77
18	91-110	SLKLNVYEEIKLRIRAEKEN	2445.1	8.68	64
19	95-114	NVYEEIKLRIRAEKENTSSW	2464.9	6.50	41
20	99-118	EIKLRIRAEKENTSSWYEVD	2465.9	4.93	34
21	103-122	RIRAEKENTSSWYEVDSFTP	2414.7	4.78	30
22	107-126	EKENTSSWYEVDSFTPFRKA	2420.7	4.78	50
23	111-130	TSSWYEVDSFTPFRKAQIGP	2315.7	6.26	41
24	115-134	YEVDSFTPFRKAQIGPPEVH	2316.7	5.55	29
25	119-138	SFTPFRKAQIGPPEVHLEAE	2252.7	5.61	28
26	123-142	FRKAQIGPPEVHLEAEDKAI	2247.8	5.63	28
27	127-146	QIGPPEVHLEAEDKAIVIHI	2207.8	4.75	30
28	131-150	PEVHLEAEDKAIVIHISPGT	2154.7	4.75	27
29	135-154	LEAEDKAIVIHISPGTKDSV	2121.7	4.63	31
30	139-158	DKAIVIHISPGTKDSVMWAL	2180.9	7.15	28
31	143-162	VIHISPGTKDSVMWALDGLS	2135.8	5.23	28
32	147-166	SPGTKDSVMWALDGLSFTYS	2161.7	3.92	33
33	151-170	KDSVMWALDGLSFTYSLLIW	2345.1	3.92	31
34	155-174	MWALDGLSFTYSLLIWKNSS	2332.1	5.95	30
35	159-178	DGLSFTYSLLIWKNSSGVEE	2244.8	3.96	44
36	163-182	FTYSLLIWKNSSGVEERIEN	2384.9	4.71	26
37	167-186	LLIWKNSSGVEERIENIYSR	2406.0	6.45	39
38	171-190	KNSSGVEERIENIYSRHKIY	2422.0	8.70	31
39	175-194	GVEERIENIYSRHKIYKLSP	2431.1	8.70	33
40	179-198	RIENIYSRHKIYKLSPETTY	2511.2	9.47	37
41	183-202	IYSRHKIYKLSPETTYCLKV	2442.3	9.42	54
42	187-206	HKIYKLSPETTYCLKVKAAL	2306.2	9.41	37
43	191-210	KLSPETTYCLKVKAALLTSW	2262.0	8.89	47
44	195-214	ETTYCLKVKAALLTSWKIGV	2226.0	8.99	46
45	199-218	CLKVKAALLTSWKIGVYSPV	2176.0	9.60	65
46	203-222	KAALLTSWKIGVYSPVHCIK	2314.1	9.60	33
47	207-226	LTSWKIGVYSPVHCIKTTVE	2260.9	8.22	28
48	210-229	WKIGVYSPVHCIKTTVENEL	2315.9	7.08	28

Which [illegible] are peptides [illegible]

Table 2 : Sequences and analytical data of overlapping human biotinylated synthetic peptides derived from the human IFNAR1 receptor protein. Peptides are numbered according to the position of the amino-terminal residue within the IFNAR1 sequence. Sequences corresponding to the epitope peptide are underlined. Binding % represents the 64G12 binding to coated peptides relative to maximum binding. Results are the means of two independent experiments.

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24/02 '00 10:17 FAX 01 [illegible]8 34 44 TOVEY
le 24/02/00 10:27 Pg:36/36
036

Peptide	Modified Sequence	M.W.	pI	Binding %
1	NFSSL	563.7	5.5	48
2	NFSSLK	693.9	9.01	41
3	NFSSLKL	807.1	9.01	43
4	NFSSLKLN	921.2	9.0	55
5	NFSSLKLNV	1020.3	9.0	52
6	NFSSLKLNVY	1183.5	8.85	100
7	KLNVYE	763.9	6.21	57
8	LKLNVYE	877.1	6.21	50
9	SLKLNVYE	964.2	6.21	61
10	SSLKLNVYE	1051.3	6.21	73
11	FSSLKLNVYE	1198.5	6.21	96
12	NFSSLKLNVYE	1312.6	6.21	98
13	NFSSLKANVYE	1270.5	6.21	43
14	NFSSLKGNVYE	1256.5	6.21	47
15	NFSSLALNVYE	1255.5	3.75	42
16	NFSSLGLNVYE	1241.5	3.75	44
17	NFSSAKLNVYE	1270.5	6.21	73
18	NFSSGKLNVYE	1256.5	6.21	65
19	NFSALKLNVYE	1296.6	6.21	98
20	NFSGLKLNVYE	1282.6	6.21	82
21	NO PEPTIDE	-	-	38

Table 3

Received 03-03-00 10:17 From-33 01 42 66 08 90 To-FOLEY & LARDNER Page 04

6. **Contribution of individual residues of the IFNAR1-epitope to the binding of the 64G12 mAb**

Analysis of the peptide sequence interacting with the 64G12 mAb, revealed a common sequence (NFSSLKLNVYE), mapping to residues 88-98 of the IFNAR1 SD1 domain. In order to determine the smallest peptide required for antibody binding, and the relative importance of each constituent amino acid involved in binding, a series of 20 chimeric biotinylated peptides were synthesized in which a single residue only was modified or deleted from one peptide to another. The following conclusions can be drawn from data presented in Table 3 and Figure 4 as to the relative importance in antibody binding of each individual residue of the sequence :

Beginning with the full sequence (NFSSLKLNVYE), the deletion of either the amino-terminal N or the carboxyl terminal E has little or no effect on antibody binding. The further deletions from the amino terminus of F decreases specific binding by about 50%, while the combined deletion of F and S further decreases binding. Any further deletion from the amino terminus lowers binding to approximately background levels. From the carboxyl terminus, the deletion of the Y reduces antibody binding to near background levels ; further deletions from this end are equivalent to the tyrosine deletion and, therefore, are uninformative. Thus, the minimum full-binding peptide is the 9-mer, FSSLKLNVY (residues 89-97). Within this sequence, the binding of 64G12 is slightly sensitive to the substitution of glycine (but not alanine) at the second serine, and is more sensitive to alanine and glycine at the first leucine. However, the K or second L appear critical for antibody binding, since their substitution by alanine or glycine decreases antibody binding to background levels. Thus, positions throughout the nonapeptide have varying effects on binding to 64G12.

7. As a conclusion, the above results clearly demonstrate that peptides or polypeptides consisting of the amino acid sequences 1-229 or 27-229 of SEQ ID NO: 1 or 2 specifically bind to the monoclonal antibody, 64G12.

8. I further declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine and imprisonment, or both under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

2-03-00.

Date

Michael Tovey

Michael G. TOVEY

OIPE JC80
MAR 0 3 2000
PATENT & TRADEMARK OFFICE

PATENT

Attorney Docket No. 017283/0123

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of :
BENOIT et al. :
: Group Art Unit: 1641
Serial No.: 09/240,975 :
: Examiner: DEVI, S.
Filed: February 2, 1999

For: MONOCLONAL ANTIBODIES AGAINST ALPHA IFN

DECLARATION OF AVAILABILITY

ASSISTANT COMMISSIONER FOR PATENTS AND TRADEMARKS
WASHINGTON, D.C. 20231

SIR:

The undersigned, a representative of MEDISUP INTERNATIONAL N.V., having a place of business at Kaya W.F.G. Mensing 14, 431 Netherlands Antilles, declares and states that:

1. I have reviewed the Assignment in the above-identified application, a copy of which is attached hereto, and I believe in good faith that MEDISUP INTERNATIONAL N.V. is the Assignee of the entire right, title and interest in and to the invention described and claimed in application Serial No. 09/240,975, filed September 3, 1993, for MONOCLONAL ANTIBODIES AGAINST THE INTERFERON RECEPTOR, WITH NEUTRALIZING ACTIVITY AGAINST TYPE I INTERFERON.

2. Cell culture 64G12 was deposited at the ECACC, PHLS CAMR, PORTON DOWN on 25th February, 1992, under accession No. 92022605.

3. Access to the culture will be available during pendency of the patent application to one determined by the Commissioner to be entitled thereto under 37 C.F.R. 1.14 and 35 U.S.C. 122.

4. All restrictions on the availability to the public of the culture so deposited will be irrevocably removed upon the granting of the patent.

5. MEDISUP INTERNATIONAL N.V. will maintain the deposited culture, and will refurnish such culture should it become non-viable while on deposit.

6. The deposited culture will be maintained at said depositary for a period of at least five years after the most recent request for the furnishing of a sample of the deposited cultures was received by the depositary, and, in any case, for a period of at least thirty (30) years after the date of the deposit, or during the enforceable life of the patent, whichever is later.

I further state that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such will full false statements may jeopardize the validity of this application or any patent issuing thereon.

March 2nd, 2000
Date

[signature]
Signature

Kevin MARTIN.
Typewritten Name

Managing Director.
Official Title

CURRICULUM VITAE

Michael G. TOVEY

PRESENT AFILIATION

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Fax (33-1) 49 58 34 44

PERSONAL

Birth :	March 8, 1945
Citizenship :	British

EDUCATION

B.s.C. 1969	Special Microbiology, London University
Ph.D. 1972	Microbiology, London University

RESEARCH AND ACADEMIC APPOINTMENTS

Sept. 1968 - Oct. 1971	Assistant (Department of Microbiology - Prof. S. Pirt) Queen's Elizabeth College, London.
1969 - 1971	Assistant in Biochemistry, Queen's Elizabeth College, London.
	Assistant in Bacteriophage genetics and animal virology, Queen's Elizabeth College, London.
1971 - April 1973	Post-doctoral research fellow of European Molecular Biology Organization at the Institut de Recherches Scientifiques sur le Cancer, Laboratory of Viral Oncology (Dr Ion Gresser), Villejuif, France.
April 1973 - Dec. 1976	Attaché de recherche I.N.S.E.R.M. at the Institut de Recherches Scientifiques sur le Cancer, Villejuif, France.
Dec.1976 - Dec. 1979	Chargé de Recherche I.N.S.E.R.M. at the Institut de Recherches Scientifiques sur le Cancer, Villejuif, France.
Jan.1980 - 1983	Maître de Recherche I.N.S.E.R.M. at the Institut de Recherches Scientifiques sur le Cancer, Villejuif, France.

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Characterization of a Domain of a Human Type I Interferon Receptor Protein Involved in Ligand Binding

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ABSTRACT

Two monoclonal antibodies that recognize different epitopes of the extracellular domain of one of the proteins that constitute the type I interferon receptor were used to delineate the interferon binding site. Antibody 64G12 both inhibits the binding of radiolabeled interferon-α_2 and IFN-α_8 to their cell surface receptors and neutralizes the antiviral and antiproliferative actions of all the type I interferons tested, including IFN-β, IFN-ω, and human leukocyte IFN, a mixture of different interferon-α isotypes. Antibody 34F10 recognizes the type I interferon receptor with an affinity similar to that of the MAb 64G12 but does not inhibit either the binding or the biologic activity of any of the type I interferons tested. Both antibodies recognize a protein of 105 ± 5 kD from either Daudi or Ly28 cells. Immunoprecipitation following surface iodination demonstrated that the neutralizing MAb recognizes a protein of 105 kD and the nonneutralizing MAb a protein of 110 kD in extracts of Daudi cells. A second less intense band was also detected by both antibodies. Cross-linking of IFN-α_2 to its receptor before immunoprecipitation prevented the neutralizing antibody from immunoprecipitating the receptor protein, but the nonneutralizing MAb was still able to recognize a 140 kD protein corresponding to the cross-linked interferon-receptor protein complex. Thus, an interferon binding domain appears to be localized in a region between amino acids 23 and 229 of the extracellular domain of a transmembrane protein that forms part of the type I interferon receptor complex containing the epitopes recognized by each antibody.

INTRODUCTION

THE INTERFERONS (IFN) are multifunctional cytokines that comprise proteins of three antigenic classes, α, β, and γ. The α interferons are encoded by a superfamily of 14 functional α genes and a single functional ω gene.[1-3] The β and γ interferons are encoded by single copy genes. Interferons exert their characteristic biologic actions, which include the establishment of an antiviral state, inhibition of cell proliferation, and modulation of the immune system, by binding to high-affinity cell surface receptors. Interferon-α, interferon-β, and interferon-ω, the so-called type I interferons, share a common receptor; interferon-γ binds to a quite distinct cell surface receptor.[4-7] The extent of the biological activity of IFN-α appears to be proportional to the number of type I receptors, as indicated by the sensitivity of human diploid fibroblasts containing one, two, or three copies of chromosome 21, which carries the gene(s) that code for the human interferon-α receptor.[8,9] The characteristic species specificity of the interferons is also determined at the level of the cell surface receptor.[1,10-13] Binding of interferon-α to its receptor is followed by the formation of an activated high-affinity IFN-receptor complex, which is a prerequisite for the subsequent development of both the antiviral and antiproliferative actions of interferon-α.[14-16] Binding of interferon-α to its receptor has been shown to result in the activation of the multimeric transcription factor ISGF3, its translocation to the nucleus, and activation of those genes that contain an interferon responsive element in their 5′-untranslated region, resulting in the establishment of the characteristic biologic actions of the interferon.[17-25]

A major component of the human type I interferon-receptor complex was recently cloned,[26] a transmembrane glycoprotein structurally related to the cytokine receptor superfamily and characterized by two tandem 200 amino acid domains, each consisting of two subdomains of equal size. The predicted folding of each of the 100 amino acid subdomains is consistent with the formation of the seven β strands (S1–7) of the immunoglobulin constant domain.[27,28] The use of IFN-α/βR% mice in

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which the gene encoding this protein has been inactivated by homologous recombination has shown that this protein plays an important role in mediating the antiviral action of the type I interferons.[29] It is becoming clear, however, that this protein constitutes one of a number of proteins associated with the human type I interferon-receptor complex.[30,31]

The study of the IFN-receptor complex has heretofore been limited by the availability of sufficient quantities of purified functional receptor. The availability of recombinant soluble receptor, as well as two monoclonal antibodies that recognize different domains of this human type I interferon receptor protein, has allowed us to identify a region from amino acids 23 to 229 containing the epitope recognized by the two anti-IFN receptor antibodies and containing a domain involved in ligand binding.

MATERIALS AND METHODS

Chemicals and reagents

All reagents for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from BioRad (South Richmond, CA). Horseradish peroxidase-conjugated sheep antimouse IgG and enhanced chemiluminescence kits (ECL) were purchased from Amersham (Little Chalfont, UK). Disulfosuccinimidyl tartrate and iodobeads were purchased from Pierce.

Human recombinant interferon-α_2 was a gift from Dr. M. Fountoulakis (Biogen, Zurich, Switzerland), and interferon-α_8 was a gift from Dr. M. Grütter (Ciba-Geigy, Switzerland). They were iodinated as described[32] to a specific activity of 3×10^6 and 1.2×10^6 cpm/pmol, respectively.

Cell lines

Daudi, a human Burkitt's lymphoma-derived cell line[33]; Ly28, a human lymphoblastoid cell line[34]; and Madin Darby bovine kidney cells (MDBK) were maintained in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS, or 15% FCS for Daudi cells), 2 mM L-glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin.

Monoclonal antibodies

The monoclonal antibodies 64G12 and 34F10 are mouse IgG$_1$ antibodies raised against recombinant proteins corresponding to the N-terminal region of the human IFN-α receptor truncated at residue 427 just before the transmembrane domain and expressed in COS cells and *E. coli*, respectively. The 64G12 antibody is a neutralizing antibody that inhibits both the binding of radiolabeled recombinant human α interferons to specific binding sites on the surface of human cells and the antiviral and antiproliferative actions of the type I interferons. The 34F10 antibody does not inhibit either the binding or biologic activities of any of the human type I interferons tested even though both antibodies exhibit similar affinities for the human IFN-α receptor (kD of 3–5 nM). The preparation and characterization of these antibodies have been described previously.[18]

SDS-PAGE and immunoblotting

SDS-PAGE was performed according to Laemmli.[35] After blotting of separated proteins onto a polyvinyldifluoride (PVDF) Millipore membrane, the membranes were saturated in 20 mM Tris, pH 7.6, 0.14 M NaCl, and 0.05% Tween 20 (TBS-T) containing 3% bovine serum albumin fraction V (Sigma) and washed three times with TBS-T. Immunoblots were performed by incubating membranes for 1 h at room temperature with TBS-T containing 1 μg/ml of affinity-purified MAb 64G12. The membranes were then washed three times with TBS-T and further incubated with horseradish peroxidase-conjugated sheep antimouse Ig (1:30,000 dilution) for 45 minutes. After a further five washes with TBS-T, the bands were visualized by ECL according to the manufacturer's specifications.

Immunoprecipitation of labeled cell surface proteins with monoclonal antibodies

Daudi cells (3×10^7) were surface labeled with ^{125}I using the iodobead method according to the manufacturer's instructions. After labeling, the cells were washed with phosphate-buffered saline (PBS) and lysed for 30 minutes at 0°C with 50 mM Tris, pH 8, 0.5% Triton X-100, 240 mM NaCl, 10% glycerol, and 0.1 mM EDTA (ethylene diamine tetraacetic acid) containing 1 mM phenyl methyl sulfonyl fluoride, aprotinin, 10 μg/ml, leupeptin, and pepstatin, 2 μg/ml. Lysates were incubated for 2 h at 4°C with a mixture of affinity-purified mouse and rabbit IgG (20 μg each), followed by the addition of protein G-Sepharose (60 μl) for 4 h at 4°C. After centrifugation, the supernatants were incubated overnight with the antibodies indicated (15 μg) and then incubated with protein G-Sepharose (60 μl) for 4 h at 4°C. After five washes the samples were subjected to 10% SDS-PAGE under reducing conditions. Dried gels were exposed to x-ray film to detect labeled proteins.

Affinity cross-linking of [^{125}I]interferon-α_2 to its receptors

Daudi cells (1.5×10^8) in 12 ml RPMI 1640 and 10% FCS were incubated with 2600 U/ml (0.3 nM) of [^{125}I]interferon-α_2 for 2 h at 4°C. The cells were washed three times in PBS, resuspended in 12 ml PBS and 1 mM MgCl$_2$, cross-linked with 0.9 mM disulfosuccinimidyl tartrate (Pierce) for 15 minutes at 0°C, and lysed at a concentration of 0.75×10^8 cells/ml in the same Triton lysis buffer as before.

Immunoprecipitation with 34F10 and 64G12 MAbs was performed as described earlier.

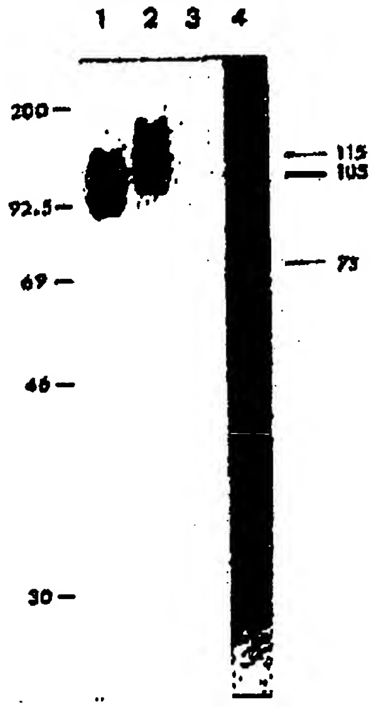

FIG. 1. Western immunoblot analysis of the interferon-α/β receptor. Plasma membranes (75 μg) from Daudi cells: lane 1, Ly28 cells, lane 2; MDBK cells, lane 3, were prepared as described in Materials and Methods. Lane 4 shows the purified glycoprotein (0.1 μg) present in the supernatant of CHO cells transfected with a cDNA encoding the extracellular domain of the human interferon-α receptor protein. Samples were electrophoresed under reducing conditions on 10% SDS-polyacrylamide gels and electrotransferred onto nitrocellulose membranes (Amersham). Specific antigens to 64G12 MAb (1 μg/ml) were detected by enhanced chemiluminescence (ECL).

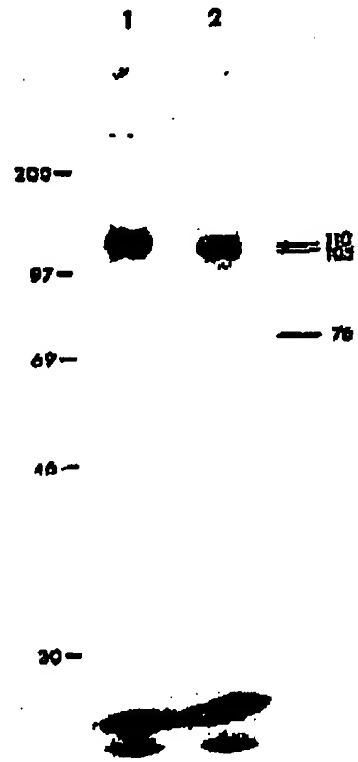

FIG. 2. The 64G12 and 34F10 MAbs recognize a major antigen of 105 and 110 kD, respectively. Another weak band of 76 kD is also recognized by both MAbs. Iodinated Daudi cells were lysed and precipitated using 15 μg of the MAb indicated. Preclearings were performed with mouse and rabbit IgG. Electrophoresis was run under reducing conditions on 10% SDS-polyacrylamide gels. Standard molecular weight protein markers are shown on the left.

RESULTS

Western blot analysis of the reactivity between MAb (64G12) and the native interferon-α receptor

Plasma membranes from the human Burkitt's lymphoma cell line Daudi, the human Epstein-Barr virus-transformed lymphoblastoid cell line Ly28, or the Madin Darby bovine kidney cell line MDBK,[37] and soluble interferon-α/β receptor were analyzed under reducing conditions by 10% SDS-PAGE[36] and electroblotted to PVDF. As shown in Fig. 1, the MAb 64G12 was found to recognize a 105 kD antigen on the surface of Daudi cells (lane 1) and a 115 kD antigen on the surface of Ly28 (lane 2). A 105 kD antigen was also detected in extracts of plasma membranes from both the human amniotic cell line WISH and the human promonocytic cell line U937 (data not shown). The antibody did not react with plasma membranes from heterologous MDBK cells (lane 3).

The antiinterferon-α receptor MAb was also found to recognize a 75 kD glycoprotein present in the supernatant of CHO (Chinese hamster ovary) cells transfected with a cDNA encoding the extracellular domain of a human interferon-α receptor protein (lane 4). All the bands detected by the MAb exhibited the characteristic diffuse form of glycoproteins. No cross-reactivity was detected when irrelevant antibodies were used as controls.

Receptor proteins detected by immunoprecipitation of surface-iodinated cells

To demonstrate the specificity of the 64G12 and 34F10 antibodies and to characterize the antigens recognized by those antibodies, Daudi cells were surface labeled with [^{125}I]iodine using Iodobeads according to the manufacturer's instructions. After iodination, cells were lysed and the immunoprecipitates obtained with both antibodies were analyzed by SDS-PAGE under reducing conditions (Fig. 2). The monoclonal antibody 34F10 precipitated a 110 kD band (lane 1), and the 64G12 MAb

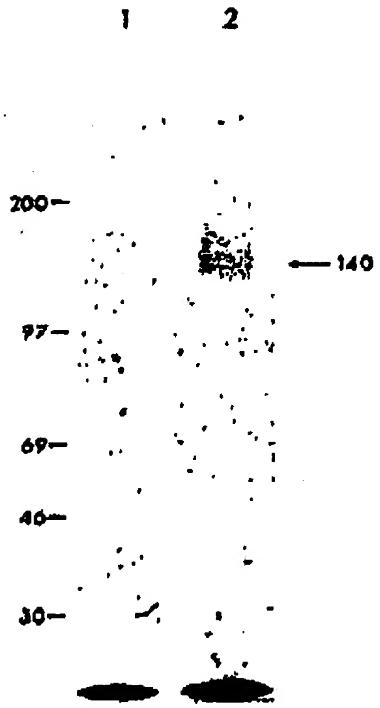

FIG. 3. [125]I-IFN-α_2 was affinity cross-linked with disulfosuccinimidyl tartrate to the cell surface receptors of Daudi cells. The lysates were immunoprecipitated with neutralizing anti-IFN receptor MAb 64G12 (lane 1) or nonneutralizing anti-IFN receptor MAb 34F10 (lane 2) and subjected to 10% SDS-PAGE under reducing conditions. Standard molecular weight protein markers are shown on the left.

precipitated a 105 kD band (lane 2), the apparent molecular weights of which correspond exactly to those determined by western blotting (see Fig. 1). An additional weak 75 kD band was detected by both antibodies upon immunoprecipitation of surface iodinated cells but not by western blotting (Fig. 1) or by immunoprecipitation of cross-linked [[125]I]interferon-α_2-receptor complexes (Fig. 3).

Precipitation of cross-linked [[125]I]interferon-α_2-receptor complexes by MAbs directed against an interferon-α receptor protein

[[125]I]interferon-α_2-receptor complexes from Daudi cells were immunoprecipitated with the neutralizing MAb 64G12 (lane 1) or the nonneutralizing MAb 34F10 (lane 2). Protein G-Sepharose was added to recover immunoprecipitated complexes. SDS-PAGE analysis (Fig. 3) shows clearly that the MAb 64G12 (lane 1) cannot bind to the native receptor when interferon-α_2 is already bound to the receptor. In contrast, when the nonneutralizing MAb 34F10 is used, a labeled interferon-receptor complex with a mobility corresponding to 140 kD was readily detected (lane 2). The apparent molecular mass corresponds to that of the natural receptor together with that of [[125]I]interferon-α_2 (20 kD). Both antibodies were unable to precipitate free [[125]I]interferon-α_2, demonstrating that they recognize an epitope on the interferon-α receptor but not the interferon protein. Both MAbs were capable of recognizing the interferon-α receptor on Daudi cells when analyzed by flow cytometry.[35] Given that the MAb 64G12 is capable of inhibiting the binding of both interferon-α_2 and interferon-α_8 to Daudi cells; these results suggest that this monoclonal antibody recognizes a region of the native interferon receptor involved in ligand binding, which would explain why interferon-α_2 and MAb 64G12 are antagonistic in their action. We emphasize that this monoclonal antibody is able to recognize the same molecule to which labeled interferon binds in cross-linking experiments, strongly suggesting that the glycoprotein detected by the 64G12 MAb is indeed an interferon binding protein.

DISCUSSION

In this study we have identified a 105 kD ($\pm$ 5 kD) antigen from the plasma membranes of both Daudi cells and Ly28 cells that is specifically recognized by two different monoclonal antibodies raised against a recombinant protein corresponding to the extracellular domain of one of the proteins that constitute the type I interferon receptor. The slight difference in mobility observed between the proteins detected on Daudi cells (105 kD) and Ly28 cells (115 kD) is probably a result of differences in posttranslational modifications of the IFN receptor in the two cell lines. It has been shown previously[35] that the primary sequence of the protein detected by the 64G12 MAb is the same in both Ly28 and Daudi cells. This led us to conclude that the differences seen by Western blotting are probably caused by differences in the glycosylation pattern of these proteins, as already shown for CD45.[36] In addition, it should be noted that the antigen detected on two other human cell lines (data not shown) is similar in size to that detected on Daudi cells (105 kD), and the higher apparent molecular weight observed on Ly28 cell line is an exception among the cell lines tested.

The 64G12 MAb is unable to recognize an epitope on heterologous bovine MDBK cells, which, although they bind human IFN-α, possess a quite distinct interferon receptor that nevertheless belongs to the same family of cytokine receptors (Fig. 1, lane 3). Furthermore, the 64G12 MAb does not recognize an epitope on mouse cells (data not shown), which do not bind human interferon and possess an interferon receptor with very little sequence homology with the human type I IFN receptor.[37,38] The bovine IFN-α receptor, however, retains its ability to bind labeled IFN-α following SDS-PAGE and transfer to an Immobilon PVDF membrane when sulfhydryl reducing agents and heating are omitted in sample preparation.[39,40] On the other hand, the 64G12 MAb prepared against the extracellular domain of the cloned IFN-α/β receptor protein produced in COS cells,[35] recognizes the extracellular region (75 kD) of the IFN-α/β receptor secreted by CHO cells transfected with the extracellular domain of the type I IFN receptor (Fig. 1, lane 4). To determine whether the two MAbs are able to recognize the

same antigen from ^{125}I-labeled cells, we immunoprecipitated labeled proteins after surface iodination of Daudi cells. Both monoclonal antibodies 64G12 and 34F10 were able to immunoprecipitate the same protein of 105 (± 5 kD). It should be noted that this molecular mass is in agreement with that observed by immunoblotting (Fig. 1).

Colamonici et al.[31,42] have described anti-IFN-α receptor monoclonal antibodies that recognize three proteins (110, 130, and 210 kD) in extracts of surface-labeled cells and in western blots, as well as after cross-linking of iodinated IFN-α_2 to the cell surface. The 110 kD protein described by Colamonici et al. (termed the α subunit) is similar in size to the major protein detected by both the 64G12 and 34F10 antibodies. Further experiments are necessary, however, to demonstrate whether these are indeed the same protein. In addition to the major antigen (105 kD) detected, another weak, 76 kD band (Fig. 2) was also recognized specifically by both MAbs. We do not know, however, whether this band is an interferon binding protein. The presence of such a band raises the question of the requirement of an accessory protein for a functional interferon receptor system.[1,8,30,35,43] Novick et al.[21] recently described an additional IFN binding protein that appears to be part of the human type I IFN receptor complex. In addition to determining the identity of this 76 kD antigen, we are currently investigating the question of why the 76 kD band is not detected by ligand blotting with IFN-α_2 or IFN-α_8 or by immunoblotting with the two monoclonal MAbs 64G10 and 34F10. The slight differences observed in the migration of the proteins detected by the two antibodies (Fig. 2) could be related to differences in the epitope recognized by the two antibodies. The 34F10 antibody was raised against nonglycosylated, *Escherichia coli*-derived, soluble IFN-α receptor protein and could therefore recognize an epitope near a glycosylation site that is not always glycosylated. The antibody 64G12 was raised against glycosylated, COS cell-derived, soluble IFN-α receptor and may consequently recognize and immunoprecipitate a more glycosylated form of the receptor. It is of considerable interest that labeled IFN cross-linked to the cell surface receptor on Daudi cells could be immunoprecipitated with the nonneutralizing MAb 34F10 but not with neutralizing MAb 64G12. The interferon-receptor complex of 140 kD that was identified is in good agreement with the mobility of a major cross-linked complex described previously.[44–47] The fact that no interferon-receptor complex is immunoprecipitated with the neutralizing MAb 64G12 indicates that the ligand (IFN) and the antagonist (MAb 64G12) are competing for a common site on the extracellular domain of this interferon receptor binding protein. Both antigens from Daudi or Ly28 cells are IFN-α binding proteins: they could be specifically cross-linked by ^{125}I-IFN-α_2 and then immunoprecipitated with the nonneutralizing MAb 34F10 but not with the antagonist-neutralizing MAb 64G12. Thus, we have shown by these experiments that a component of the ligand binding site of the human interferon-α receptor is localized at least partly within the first half of the molecule (amino acids 23–229), determined by the ability of the MAbs to recognize recombinant proteins corresponding to truncated fragments of the extracellular domain of the IFN receptor protein (D. Maguire and I. Plavec, unpublished results). Work is in progress using overlapping synthetic peptides to delineate further the precise position of the epitopes recognized by both antibodies. The region recognized by both monoclonal antibodies corresponds to the first of the two repeated domains, which are homologous to the type II interferon receptor.[24–28,48,49] The results of our experiments, however, do not exclude the possibility that the membrane-proximal (second) domain is not involved in ligand binding.

In conclusion, in this study we have delineated (by the use of two monoclonal antibodies) a component of the ligand binding site on the extracellular domain of an interferon-α/β receptor protein. Determination of the precise organization of this ligand binding domain, however, must await crystallization of the purified soluble interferon binding protein, alone or complexed with interferon, and subsequent x-ray diffraction studies.

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